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Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord

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Transplantation approaches using cellular bridges[1,2], fetal central nervous system cells[3-5], fibroblasts expressing neurotrophin-3 (ref. 6), hybridoma cells expressing inhibitory protein-blocking antibodies[7], or olfactory nerves ensheathing glial cells[8] transplanted into the acutely injured spinal cord have produced axonal regrowth or functional benefits. Transplants of rat or cat fetal spinal cord tissue into the chronically injured cord survive and integrate with the host cord, and may be associated with some functional improvements[9]. In addition, rats transplanted with fetal spinal cord cells have shown improvements in some gait parameters[10], and the delayed transplantation of fetal raphe cells can enhance reflexes[11]. We transplanted neural differentiated mouse embryonic stem cells into a rat spinal cord 9 days after traumatic injury. Histological analysis 2–5 weeks later showed that transplant-derived cells survived and differentiated into astrocytes, oligodendrocytes and neurons, and migrated as far as 8 mm away from the lesion edge. Furthermore, gait analysis demonstrated that transplanted rats showed hindlimb weight support and partial hindlimb coordination not found in 'sham-operated' controls or control rats transplanted with adult mouse neocortical cells.

Neural progenitors isolated from the adult central nervous system differentiate into neurons and glia after transplantation into brain[12], and differentiate into oligodendrocytes and astrocytes after transplantation into spinal cord (F. Gage, personal communication). Another source of undifferentiated cells is embryonic stem (ES) cells, genetically normal immortal cells that have been derived from several species, including mouse and human, and are capable of differentiation into neurons and astrocytes after being transplanted into the brain[13-14]. In our first two series of studies, we induced thoracic spinal cord injury in 22 adult female Long-Evans rats by means of a 10-gram rod 2.5 mm in diameter, falling 25 mm (refs. 15,16). We used ES cell embryoid bodies derived from the D3 line[17] at the 4–/4+ stage (4 days without, then 4 days with retinoic acid) for transplantation. We transplanted partially trypsinized embryoid bodies as cell aggregates into the syrinx that formed 9 days after spinal cord contusion. We handled sham-operated control rats identically, including treatment with cyclosporine, but in place of cell transplantation, they received intra-syrinx injections of culture medium alone ($n = 11$). Beginning on the day of transplantation, all rats received cyclosporine daily to prevent rejection. Hindlimb motor function was assessed using the Basso–Beattie–Bresnahan (BBB) Locomotor Rating Scale[15]. Another group of 11 rats (plus 11 sham-operated control rats) underwent the same transplantation procedure, but using ROSA26 ES cells, a mouse ES cell line containing the *lacZ* transgene and expressing β-galactosidase (β-gal), and rats were killed 2 weeks after transplantation for histology and quantitative cell counting.

Mouse ES cell-derived cells marked genetically (using the ROSA26 line) and pre-labeled *in vitro* with a 24-hour pulse of 10 μM

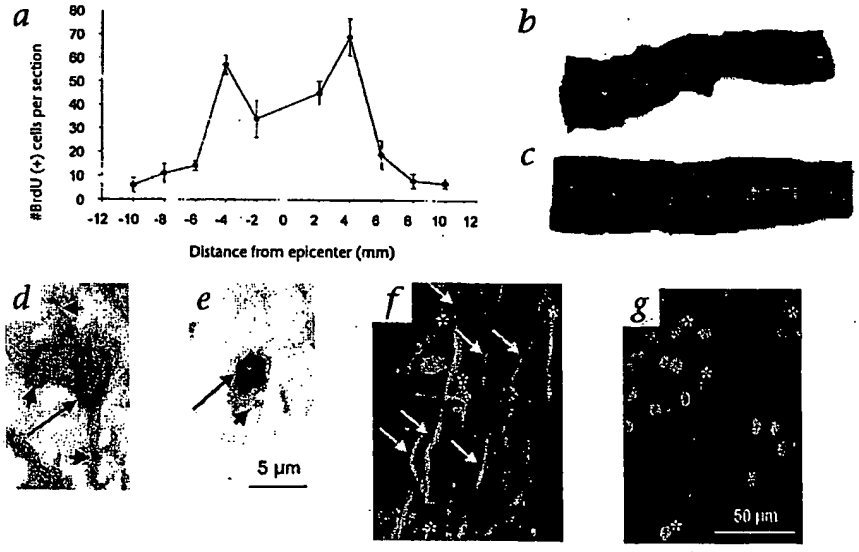

Fig. 1 BrdU labeled ES cell-derived cells 2 weeks after transplantation. *a*, BrdU-labeled nuclei per 1-mm segment in longitudinal sections ($n = 11$ rats; three sections per rat). Data represent mean ± s.e.m. *b* and *c*, Hoechst 33342-labeled sections 42 d after injury, transplanted with vehicle (*b*) or ES cells (*c*) 9 d after injury. *d*, BrdU-positive cell (purple; long arrow) co-labeled with GFAP (brown; short arrows). *e*, BrdU-labeled cell (purple; long arrow) co-labeled with APC CC-1 (brown; short arrow). *f* and *g*, The mouse-specific marker EMA shows processes (arrows) emanating from ES cells (*f*). *, Nuclei in *f* are stained by Hoechst 33342 in *g*.

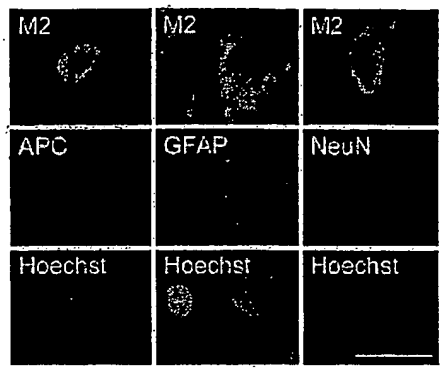

Fig. 2 Transplanted ES cell-derived cells differentiate into oligodendrocytes, astrocytes and neurons. Top row, immunostaining using an antibody against M2 that selectively labels mouse cells, but not rat (identifies transplanted ES cells); middle row, phenotype-specific immunostaining to recognize oligodendrocytes (APC), astrocytes (GFAP) and neurons (NeuN); bottom row, Hoechst 33342 nuclear DNA labeling. Samples are from rats 2 weeks after transplantation; one column represents one cell with three different labels. Scale bar represents 10 μm.

BrdU could be identified *in situ* 14–33 days after being transplanted; identification could also be achieved with the mouse-specific antibodies M2 (ref. 18), EMA (ref. 19) or Thy 1.1/1.2 (data not shown for Thy 1.1/1.2). At 2–5 weeks after transplantation, ES cell-derived cells were found in aggregates or dispersed singly throughout the injury site; furthermore, single cells could be found as far as 8 mm away from the syrinx edge in either the rostral or caudal direction (Fig. 1). In most of the transplanted rats, by 2 weeks after transplantation, ES cell-derived cells filled the space normally occupied by a syrinx in medium-treated rats. By 5 weeks, the density of ES cell-derived cells in this area was reduced and replaced with an extracellular matrix containing fibers positive for Thy 1.1/1.2 labeling. The other mouse-specific markers, M2 and EMA, offered advantages over the genetic and DNA markers (which only mark cell bodies) in that they also labeled ES cell-derived processes, which were abundant in ES cell-transplanted rats, but were not present in sham-operated rats (Fig. 1).

Surviving ES cell-derived cells, labeled with antibodies against mouse-specific markers or BrdU, also labeled with antibodies against markers specific for oligodendrocytes (adenomatous polyposis coli gene product, APC CC-1), astrocytes (glial fibrillary acidic protein, GFAP) and neurons (neuron-specific nuclear protein, NeuN) (Figs. 1 and 2); nuclei could be identified distinctly with Hoechst 33342 staining. Most surviving ES cell-derived cells were oligodendrocytes (43 ± 6% of BrdU-labeled cells were O1-labeled; $n = 11$ rats) and astrocytes (19 ± 4% were GFAP-labeled), but some ES cell-derived neurons (8 ± 5% were NeuN-labeled) were also present in the middle of the cord (Fig. 2). Many of the ES cell-derived oligodendrocytes were also immunoreactive for myelin-basic protein, an integral component of myelin (data not shown). There was no evidence of tumor formation.

Performance in 'open field locomotion' was enhanced by ES cell transplantation (Fig. 3). In contrast to the inability of the sham-operated transplantation group to support weight with their hindlimbs, rats transplanted with ES cells demonstrated partial weight-supported ambulation. A statistical difference in BBB scores was achieved by 2 weeks after transplantation (Fig. 3*a*). After 1 month, there was a difference of two points on the BBB scale between groups: 7.9 ± 0.6, sham-operated (vehicle transplantation); 10.0 ± 0.4, ES cell transplantation. The former score indicates a gait characterized by no hindlimb weight-bearing and no coordinated hindlimb movements, whereas the latter score indicates a gait characterized by partial hindlimb weight-bearing and partial hindlimb coordination.

To assess the possibility that a 'rat-versus-mouse' immune response could contribute to the behavioral benefit, we did a third experimental series. We transplanted rats with 4–/4+ ES cells (ROSA26 line) 9 days after injury and compared them directly with two control groups: culture medium injection and transplantation of adult mouse neocortical cells ($n = 6$ per group). Immunohistologic examination of the spinal cords 5 weeks after transplantation, using antibodies directed against microglia/macrophages (CDIIb) and gamma interferon, showed that all three groups had a similar degree of inflammation (data not shown). Improved locomotor function, as assessed with the BBB locomotor scale (with assignments made using slow-motion video), was again associated only with ES cell transplantation (Fig. 3*b*).

In summary, our study demonstrates that mouse ES cell-derived cells, when transplanted into the spinal cord 9 days after weight-drop injury, survive for at least 5 weeks; migrate at least 8 mm away from the site of transplantation; differentiate into astrocytes, oligodendrocytes and neurons without forming tumors; and produce improved locomotor function. Behavioral recovery similar in magnitude to that shown here has previously only been shown in acute injury models[3–6]. The BBB locomotor score differences between transplanted and control rats were not in the portion of the scale sensitive to forelimb–hindlimb coordination, so this study did not address whether functional connections improved across the lesion site. Further study will be needed to determine the factors responsible for the benefits seen here. One possibility is enhancement of myelination. This is consistent with the rapidity of observed locomotor improvement (2 weeks) and the observation that most ES cell-derived cells were oligodendrocytes, many immunoreactive for myelin basic protein. Transplantation of oligodendrocytes or oligodendrocyte progenitors into demyelinating

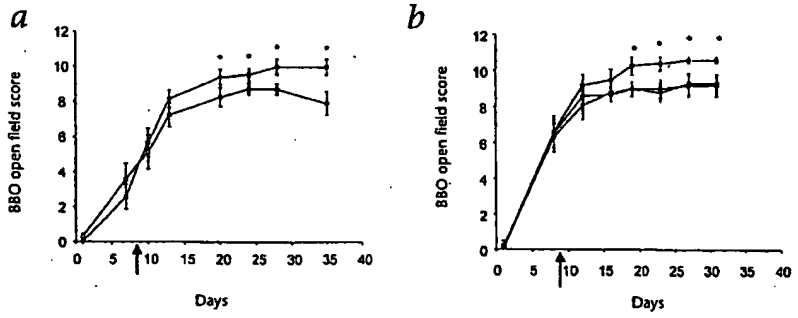

Fig. 3 ES cell-derived cell transplantation improved behavioral recovery. *a*, Change in BBB scores with transplantation, for, ES cell transplant group (●) and vehicle-treated group (○); $n = 11$ per group. *, $P < 0.05$ compared with control at same time point, repeated measures ANOVA with Tukey's test. *b*, Similar experiment to that in *a*, comparing transplantation of ES cells (●), vehicle (○) or adult mouse neocortical cells (◆); $n = 6$ per group. *, $P < 0.05$, ES cell transplantation group compared with both control groups. Data represent mean ± s.e.m. Arrows, transplantation.

chemical lesions can be associated with remyelination and improved axonal conduction[20]. Other possibilities include the reduction of delayed oligodendrocyte death, or the enhancement of host axonal regeneration (for example, by providing a favorable substrate for regrowth, or by producing growth factors).

Methods

Cell culture. D3 (from D.I.Gottlieb) or ROSA26 (from E.J. Robertson) mouse ES cells were maintained and differentiated in culture according to the published 4–/4+ protocol[17]. Undifferentiated ES cells were propagated in the presence of leukemia inhibitory factor (Life Technologies). Cells were cultured as embryoid bodies in the absence of leukemia inhibitory factor for 4 d, then treated for 4 d with retinoic acid (all-*trans*-RA, 500 nM; Sigma). On the ninth day, embryoid bodies were partially trypsinized for 5 min at 37 °C with 0.25% trypsin plus EDTA, and were resuspended in ES cell media[17] before being transplantated.

Spinal cord injury. Impact injury was induced using the weight-drop device developed at New York University ('NYU impact model') as described[15,16]. Adult Long Evans female rats (275 ± 25 g in body weight; Simonsen Lab, Gilroy, California) were anesthetized with pentobarbital (50 mg/kg, intraperitoneal), a laminectomy was done at T9–10 level, and the dorsal surface of the cord was subjected to a weight-drop impact, using a 10-gram weight (2.5 mm diameter) dropped at a height of 25 mm (ref. 16). During surgery, the rectal temperature was maintained at 37.0 ± 0.5 °C by a thermostatically-regulated heating pad (Versa-Therm 2156; Cole-Parmer, Chicago, Illinois), and during recovery, rats were placed overnight in a temperature- and humidity-controlled chamber (Thermocare, Incline Village, Nevada).

Transplantation. BBB scores were obtained the day before transplantation (day 8 after injury), control and experimental groups were matched, and rats were assigned randomly to groups, to ensure that initial locomotor scores were equalized between groups. We chose the weight-drop injury level based on previous experience with the 'NYU impact' model, to produce spontaneous recovery at a BBB score of 8, the most sensitive portion of the scale corresponding to absent weight-supported walking. At 9 d after impact injury, rats received transplants of neural differentiated ES cells (approximately 1×10^6), vehicle medium, or 1×10^6 adult mouse neocortical cells, by means of a spinal stereotaxic frame, a glass pipette with a tip 100 µm in diameter configured to a 5-µl Hamilton syringe, and a Kopf microstereotaxic injection system (Kopf Model 5000 & 900; Kopf, Tujunga, California).The ES cell or mouse neocortical cell suspension (5 µl) or vehicle medium (5 µl) was injected into the center of the syrinx at the T9 level over a 5-minute period. Three independent experiments, with time-matched controls, were completed in total. The first series was completed for behavioral analysis and late histologic analysis ($n = 11$ per group): 5 weeks after transplantation; D3 ES line transplantation compared with vehicle medium control. The second series was used to compare early (2 weeks after transplantation) and late (5 weeks after transplantation) histological outcomes ($n = 11$ per group): ES cell transplantation (ROSA *lac-Z* transgene line) compared with vehicle medium control. In the third series, three groups were compared for behavioral outcome to assess the effects of rat immune reactions to mouse cells ($n = 6$ per group): neural differentiated ES cell transplantation (ROSA26 *lac-Z* transgene line) compared with mouse neocortical cell transplantation compared with vehicle medium control; survival to 5 weeks after transplantation. All groups received the same daily cyclosporine immunosuppression (10 mg/kg subcutaneously).

Animal care. All surgical interventions and animal care were provided in accordance with the Laboratory Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, publication number 78–23, revised 1978) and the *Guidelines and Policies for Rodent Survival Surgery* provided by the Animal Studies Committee of Washington University School of Medicine. Bladders were manually expressed three times daily until reflex bladder emptying was established. Cyclosporine (10 mg/kg, subcutaneously) was administered daily to all rats in every group beginning the day of transplantation.

Behavioral testing. Behavioral testing was done weekly using the BBB Locomotor Rating Scale[15] by two individuals 'blinded' to rat treatment status. Behavioral outcomes and examples of specific BBB locomotor scores were recorded using digital video.

Immunochemistry. Primary antibodies used were directed against the following antigens (using the following dilutions): astrocytes (GFAP rabbit polyclonal, 1:4; Incstar, Stillwater, Minnesota); oligodendrocytes (APC CC-1 mIgG_1, 1:400; Calbiochem Oncogene Sciences, La Jolla, California); neurons (NeuN mIgG_1, 1:500; Chemicon, Temecula, California); mouse EMA rat hybridoma (1:1; from A.L. Pearlman, Washington University; ref. 19); mouse M2 rat hybridoma (from C. Lagenaur, University of Pittsburgh)(ref. 18); BrdU mIgG_1 or rat polyclonal (1:400; Boehringer); β-galactosidase mIgG (1:5,000; Promega). Species-specific secondary antibodies (1:200 dilution) were conjugated to Cy3, fluorescein isothiocyanate (Jackson Immunoresearch, West Grove, Pennsylvania) or Alexa 488 (1:200 dilution; Molecular Probes, Eugene, Oregon), and sections were counterstained with Hoechst 33342. Control slides lacking primary or secondary antibodies were analyzed with each series.

Cell quantification. Surviving BrdU-positive ES cells and those double-labeled for markers of differentiated neural cells were counted in three longitudinal sections, centered at the middle of the cord and separated by 200 µm, and results were averaged per rat.

Acknowledgments

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